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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C07K 5/00, 7/06, A61K 37/64

A1

(11) International Publication Number: WO 89/07609

(43) International Publication Date: 24 August 1989 (24.08.89)

(21) International Application Number: PCT/US89/00163

(22) International Filing Date: 23 January 1989 (23.01.89)

(31) Priority Application Number: 158,337

(32) Priority Date: 22 February 1988 (22.02.88)

(33) Priority Country: US

(60) Parent Application or Grant
(63) Related by Continuation
US 158,337 (CON)
Filed on 22 February 1988 (22.02.88)

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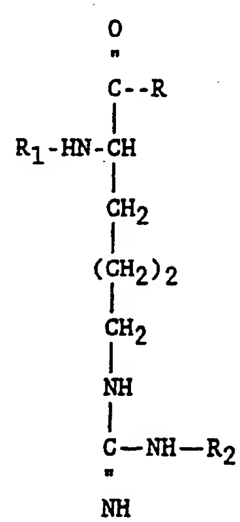
(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: HOMOARGININE-BASED PEPTIDES AS PLATELET AGGREGATION INHIBITORS



(I)

(57) Abstract

Homoarginine-based peptides of formula (I), where R, R_1 and R_2 are defined in the specification are compounds having warm-blooded platelet aggregation inhibition drug properties, e.g., N-[N-[N-[N6-(aminoiminomethyl)-N2-glycyl-L-lysyl]glycyl]-1-α-aspartyl]-L-serine, or which are chemical intermediates to such drug compounds.

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HOMOARGININE-BASED PEPTIDES AS PLATELET AGGREGATION INHIBITORS

INTRODUCTION

This invention relates to new pentapeptide compounds which have significant platelet aggregation inhibitory properties. More particularly this invention provides new pentapeptides which are useful as platelet aggregation inhibitors and intermediates thereto;

BACKGROUND OF THE INVENTION

The activity of some peptides to inhibit cell adhesion to Petri dishes and to biomatrix substrates has been published. See, e.g., Pierschbacher et al., Nature, 309, 30-3 (1984); Pierschbacher et al., PNAS, 81, 5985-88 (1984). A further partial list of papers which describe the cell adhesive properties of various peptides include:

Ginsberg et al., JBC, 260, 3931-6 (1985);

Haverstick et al., Blood, 66, 946-53 (1985);

Akiyama et al., JBC, 260, 10402-5 (1985)

as well as the following publications dealing with platelet aggregation inhibition properties of some other compounds:

Gartner et al., JBC, 260, 11891-4 (1985);

Plow et al., PNAS, 82, 8057-61 (1985);

Kloczewiak et al., Howiger, Biochemistry, 23, 1767-74 (1984).

None of the above publications discloses the new homo-arginine-based pentapeptides of this invention and their use as platelet aggregation inhibition drug compounds.

OBJECTIONS OF THE INVENTION

It is an object of this invention to provide some new pentapeptide compounds.

It is another object of this invention to provide new pentapeptide derivative compounds which have platelet aggregation inhibition drug properties, or which are useful as chemical intermediates to make such drug compounds.

Other objects, aspects and advantages of this invention will be apparent from the remaining specification which follows.

SUMMARY OF THE INVENTION

Briefly, this invention provides compounds of Formula I (see the STRUCTURES sheet, infra) (and intermediates therefor) where

R is selected from the group consisting of

-Gly-Asp-Ser-OH,

R_1 is selected from the group consisting of H-Gly, C_1 to C_3-

alkyl-C(O)-O-Gly and tert-butyloxycarbonyl; and

R_2 is hydrogen or p-toluenesulfonyl, or an acid addition salt thereof, for extraction and purification purposes, and pharmaceutically acceptable salts of the end product compounds for drug composition formulation purposes.

End product compounds within the scope of Formula I include

(1) a compound of Formula (I) and (II) wherein

R is Gly-Asp-Ser-OH;

R_1 is H-Gly, and

R^2 is hydrogen so that the resulting compound is one which can be named by formal nomenclature methods as N-[N-[N-6-(aminoiminomethyl)-N2-glycyl-L-lysyl]glycyl]-L-α-aspartyl-L-serine, or a pharmaceutically acceptable salt thereof; and

(2) a compound of Formula (I) and (III) wherein

R is Gly-Asp-Ser-OH.

R_1 is CH_3-C(O)-Gly, and

R_2 is hydrogen, so that the compound can be named N-[N-[N-[N2-(N-acetylglycyl)-N6-(aminoiminomethyl)-L-lysyl]glycyl]-L-α-aspartyl]-L-serine, or a pharmaceutically acceptable salt thereof.

Useful new chemical intermediate compounds per se within Formula I include:

(3) a compound of Formulas (I) and (III) wherein

R is hydroxyl (-OH),

R_1 is tert-butoxycarbonyl, and

R_2 is p-toluenesulfonyl, so that the compound is N2-[(1,1-dimethylethoxy)carbonyl]-N6-[imino[[(4-methylphenyl)sulfonyl]amino]-methyl]-L-serine, or a salt thereof, and

(4) a compound of Formulas (I) and (V) wherein

R is hydroxyl;

R_1 is tert-butyloxycarbonyl, and

R_2 is hydrogen, so that the compound can be named as N2-[(1,1-dimethylethoxy)carbonyl]-N6-[imino[[(4-methylphenyl)sulfonyl]amino]-methyl]-L-lysine or a salt thereof.

EMBODIMENTS OF THE INVENTION

The peptide (amino acid) compounds of this invention are generally sufficiently water soluble by themselves to enable one to dissolve them as such from a powder or crystal form into any desired physiological isotonic saline or sugar or other aqueous vehicle

administration form for introduction into a warm-blooded animal body being treated. Or they can be formulated per se into pharmaceutical aqueous-based solution formulations for administration as needed. However, since these peptide compounds are amino acids, they can be prepared and used, if desired, in the form of pharmacologically acceptable salts. Salts of the carboxylic acid compounds of this invention can be made with any pharmacologically acceptable cation such as the pharmacologically acceptable metal cations such as sodium, potassium, calcium, magnesium, as well as aluminum, zinc and iron, and the ammonium, amine and quaternary ammonium cations. Examples of pharmacologically acceptable amine cations for such pharmaceutically acceptable salts include the methylamine, dimethylamine, trimethylamine, ethylamine, dibutylamine, triisopropylamine, N-methylhexylamine, decylamine, dodecylamine, allylamine, crotylamine, cyclopentylamine, dicyclohexylamine, 1,4-diaminocyclohexane, benzylamine, diethylenetriamine, and the like, aliphatic cycloaliphatic, araliphatic amines containing up to and including about eighteen (18) carbon atoms, as well as salts with heterocyclic amines, e.g., piperidine, morpholine, pyrrolidine, piperazine, and the C_1 to C_3-lower-alkyl derivatives thereof, e.g.,

1-methylpiperidine,
4-ethylmorpholine,
1-isopropylpyrrolidine,
2-methylpyrrolindine,
1,4-dimethylpiperazine,
2-methylpiperidine,

and the like, as well as amines containing water-solubilizing or hydrophilic groups, e.g.,

mono-, di-, and triethanolamine,
ethyldiethanolamine,
N-butylethanolamine,
2-amino-1-butanol,
2-amino-2-ethyl-1,3-propanediol,
2-amino-2-methyl-1-propanol,
tris(hydroxymethyl)aminomethane,
N-phenylethanolamine,
N-(p-tert-amylphenyl)diethanolamine,
glactamine,

N-methylglycamine,
N-methylglucosamine,
ephedrine,
phenylephrine,
epinephrine,
procaine,

and the like. Further useful amine salts are the basic amino acid salts, e.g.,

lysine and
arginine.

Examples of suitable pharmacologically acceptable quaternary ammonium cations are

tetramethylammonium,
tetraethylammonium,
benzyltrimethylammonium,
phenyltriethylammonium, and the like.

Pharmaceutically acceptable acid addition salts are formed at the heterocyclic amine moiety and are also useful for administering the compounds of this invention. These salts include hydrochloride, hydrobromide, hydroiodide, p-toluene-sulfonate, sulfate, phosphate, acetate, propionate, lactate, maleate, malate, succinate, tartrate, and the like. They are prepared by methods well know in the art.

The useful end product compounds of this invention are compounds of Formula I having the Gly-Asp-Ser-OH group in the R position, hydrogen or Gly and a C_2 to C_4-carboxylic acyl in the R_1 position, e.g., acetyl, propionyl, n-butanoyl, isobutanoyl, tert-butanoyl, and hydrogen in the R_2 position.

The compounds of this invention can be prepared in progressive steps starting from homoarginine. Homoarginine can be purchased commercially. Homoarginine (Har) containing peptides or proteins can be obtained by guanidination of lysine residues already present at the desired location with O-methylisourea [see Habeeb, A.F.S.A., Biochem Biophys. Acta., 34, 294-296 (1959); Epaud, R. M. et al., Arch. Biochem. Biophysics., 154, 132-136 (1973); and Bregman, M.D. et al., J. Biol. Chem., 255, 11725-11731 (1980)] or with 1-guanyl-3,5-dimethylpyrrazole [see Habeeb, A.F.S.A., Can. J. Biochem. and Physiol., 38, 493-501 (1960); Bodanszky, M. et al., J. Amer. Chem. Soc., 86, 4452-4459 (1964); Zaoral, M. et al., Coll. Czech. Chem.

Commun., 37, 3350-3351 (1972) and E. Gunnar G. Lindberg et al., Int. J. Peptide Protein Res., 8, 193-198 (1976)] or by variations of these procedures [see Bannard, R.A. et al., Can. J. Chem., 36, 1541-1549 (1958) and Ross, J.B.A. et al., Biochim Biophys. Acta., 576, 372-384 (1979). The disadvantage of those published methods include lack of complete reaction [see E. Gunnar G. Lindberg et al., supra] and lack of specificity when more than one lysine or other reactive functionalities (e.g., terminal amine, tyrosine) are present.

This invention enables preparation of a stable pentapeptide solid and protected derivative of homoarginine (Har) that allows greater synthetic diversity.

The compounds of this invention can be prepared using the convenience of solid phase peptide synthesis (SSPS) which procedure allows the rapid production of analogs of natural protein fragments or peptides containing unnatural amino acids for biological evaluation [see Merrifield, R.B., Biochemistry, 3, page 1385 et seq. (1964)]. We have discovered that the incorporation of homoarginine by the SSPS method eliminates the problem of non-specificity of the guanidination of lysine or side reactions with other unprotected amino acids inherent in this method.

First, the alpha-amino group is protected by reaction with reagent [2-(tert-butyloxy carbonylimino)-2-phenylacetonitrile] which is followed by reacting the guanidino group [-NH-C(NH)-NH_2] of homoarginine with p-toluenesulfonyl chloride in pH 11 to 12 aqueous acetone mixture for about three hours.

The compounds of this invention are made in stepwise fashion starting with homoarginine which can be purchased. All amino acids except homoarginine were purchased from Peptides International. Homoarginine and all other chemicals were purchased from the Aldrich Chemical Co. or were reagent grade or better if from other sources. All HPLC solvents contained 0.1% trifluoroacetic acid. Purity was evaluated on a Varian analytical HPLC equipped with a Vydac C-18 (1/4" x 25 cm) column and eluted 1.5 ml./min with a standard 20 min linear gradient: 100/0 to 20/80: H_2O/CH_3CN_0 V/V percent.

General Procedure for Peptide Coupling for each amino acid added to the peptide chain.

1. Four washes with DCM
2. TFA wash (two minutes)

3. TFA deprotection (20 minutes)
4. 3X washes with DCM
5. 2X DIPEA neutralization (two minutes each)
6. 4X washes with DCM
7. Qualitative Ninhydrin Test.
8. HOBT/DCC coupling
   3x excess of amino acid to be added
   3x excess of HOBT
   2.5x excess of DCC (0.5M solution in DCM)
9. Qualitative Ninhydrin Test
10. 3X washes with DCM
11. 3X washes with EtOH

Solvents and Solutions

DCM - Dichloromethane
TFA - 45% TFA, 3% anisole in DCM
DIPEA - 10% diisopropylethylamine in DCM
HOBT - 1-hydroxybenzotriazole
DCC - dicyclohexylcarbodiimide

General Procedure for Acetylation (capping)

The peptide on resin was prepared with 3 DCM washes. Then to 1.5 meq of peptide on resin acetic anhydride (30 ml. of 5% Ac2O in DCM) and DIPEA (1.5 meq) was added and stirred at room temperature for two hours. The resin was checked for completeness of reaction by the ninhydrin test (14, 16). When the reaction was complete the resin was washed three times with DCM.

General High Vacuum HF Cleavage Procedure

The dried resin was placed in a fluorocarbon resin (Teflon®) reaction vessel with 1 ml. of anisole and a magnetic stir bar. Approximately 10 ml. of HF was added to the reaction vessel and the mixture was allowed to stir at 0°C. for one hour. The HF and anisole was distilled off in high vacuum. The resin was triturated with ether and the peptide was dissolved off the resin with 50% aqueous HOAc. The acetic acid was removed by rotary evaporation and the crude peptide was lyophilized from water.

The stepwise process can follow the PROCESS OUTLINE set forth hereinbelow. In step (1) the homoarginine is treated with an amino-blocking reagent such as Boc-ON [2-(tert-butyloxycarbonylimino)-2-phenylacetonitrile] under basic pH conditions in an aqueous dixoane

mixture until reaction is essentially complete, say for 20 to 24 hours. After work-up of the mixture the tert-butyloxycarbonyl-protected homoarginine can be obtained as an essentially white powder and processed further without further purification.

In step (2) the tert-Boc-homoarginine can be treated to protect the quanidino moiety and [H_2N-((NH)-NH)-] of the tert-butyloxy carbonyl-protected homoarginine by deprotonating it at a basic pH, e.g., pH 11 to 12, in a suitable diluent such as aqueous acetone with an amino-protecting reagent such as p-toluenesulfonyl chloride for time sufficient to react with essentially all of the amino nitrogen, e.g., about three hours. The resulting reaction mixture can be processed to separate the desired product, tert-Boc-homoarginine-(N^G-tosyl), from small amounts of side reaction products such as (N^G-Tos)Har and trace amounts of unreacted starting material. Preparative HPLC procedures can be used to obtain the step (2) di-protected amino acid as an essentially pure material. We noted that the retention time of homoarginine in the amino acid analyzer is slower than that of arginine. So the analyzer program was lengthened 15 minutes to allow elution of the amino acid.

In step (3) the tert-butyloxycarbonyl-homoarginine-(N^G-Tosyl) product of step (2) (the di-protected homoarginine) can then be processed by solid phase peptide synthesis procedures on a resin such as a Merrifield resin, or other resins known for use in the peptide synthesis art, following the above-outlined general procedure for amino acid coupling to build up the desired length of peptide. In this step (3) the di-protected homo-arginine mixed with the resin and then successively treated with amino acids to form the Gly-Har-Gly-Asp-Serine pentapeptide which if desired can than be removed from the resin by hydrogen fluoride cleavage procedures. This can be followed by preparative HPLC purification procedures and amino acid analyses.

In an optional step to prepare a N-acylated Gly-Har-Gly-Asp-Ser product, step (4), the Gly-Har(Tos)-Gly-Asp(Chxl)-Ser(OBzl)-Merrifield resin material, where Chxl means a cyclohexylester-protecting group and Bzl means a benzyl-protecting group can be carboxyl-group acylated. e.g., with a C_2 to C_4-alkanoic acid anhydride such as acetic anhydride, propionic anhydride, butanoic anhydride, preferably with acetic anhydride to cap the glycine amino nitrogen as desired hereinabove. The resulting capped pentapeptide is cleaved from the

resin by hydrogen fluoride (hydrofluoric acid) procedures, separated and purified for preparation for drug test and formulation purposes.

The peptides of steps (3) and (4) were tested by the following procedure to determine their blood platelet aggregation inhibition properties.

Each compound was dissolved in normal saline (0.9% sodium chloride in water solution) and added to platelet-rich plasma (PRP) to a final concentration of 100 micrograms/ml. A predetermined concentration of adenosine diphosphate (ADP) was added and the extent of aggregation was determined. Separately, a Control aggregation test was done after addition of a similar volume of normal saline to PRP. If the extent of aggregation by the test compound was decreased from the amount of aggregation in the control test, the aggregation was repeated using a lower concentration of the test peptide compound. In this manner a concentration of test peptide compound which caused a 50% inhibition of platelet aggregation was determined, the IC_{50} value. Peptides that did not inhibit aggregation at 100 micrograms/ml. were considered inactive, or at least not of interest for more advanced testing.

In this test the product of step (3), Example 3 herebelow, Gly-Har-Gly-Asp-Ser, gave an IC_{50} value of 20.

Compounds that inhibit platelet aggregation are useful for the prevention of arterial thrombosis arising from the deposition of platelets and white cells at sites of endothelial injury. Accumulation of such deposits can lead to several clinical symptoms such as (1) the deposit (thrombus) may become large enough to obstruct the flow of blood to distal regions resulting in depletion of oxygen to cells. If the affected distal regions involve vital organs such as the brain, heart or kidney, it may result in stroke, heart attack or renal failure. (2) Similar consequences may result if the thrombus formed in a large vessel embolizes and the embolus lodges in the smaller cerebral, coronary or renal vessels. Finally, (3) a thrombus may become organized and grow into an atherosclerotic plaque. These plaques may become large enough to block blood circulation. Compounds such as those described and claimed here to inhibit blood platelet aggregation could be of significant drug importance, especially to people and warm-blooded animals who are prone to thrombosis. The above test results indicate that these compounds

could be of further interest for more advance testing as blood platelet aggregation inhibitor drug compounds.

These compounds are useful whenever it is desired to inhibit platelet aggregation, reduce the adhesive character of platelets, and remove or prevent the formation of thrombi in mammals, including man, rabbits, dogs and rats. For example, these compounds are useful in the treatment and prevention of myocardial infarcts, to treat and prevent post-operative thrombosis, to promote patency of vascular grafts following surgery, and to treat conditions such as athero-sclerosis, arteriosclerosis, blood-clotting defects due to lipemia and other clinical conditions in which the underlying etiology is associated with lipid imbalance or hyperlipidemia. For these purposes, these compounds are administered orally or systemically, e.g., intravenously, subcutaneously, intramuscularly, and in the form of sterile implants for prolonged action. For rapid response especially in emergency situations, the intravenous route of ad-ministration is preferred. Doses in the range about 0.005 to about 20 mg. per kg. of body weight per day are used, the exact dose depending on the age, weight and condition of the patient or animal and on the frequency and route of administration.

These compounds are further useful as additives to blood, blood products, blood substitutes or other fluids which are used in artificial extracorporeal circulation or perfusion of isolated body portions, e.g., limbs and organs, whether attached to the original body, detached and being preserved or prepared for transplant or attached to a new body. During these circulations and perfusions, aggregated platelets tend to block the blood vessels and portions of the circulation apparatus. This blocking is avoided by the presence of these compounds. For this purpose, the compound is added gradual-ly or in single or multiple portions to the circulating blood, to the blood of the donor animal, to the perfused body portion, attached or detached, to the recipient, or to two or all of these at a total steady state dose of about 0.001 to 10 mg. per liter of circulating fluid. It is especially useful to these compounds in laboratory animals, e.g., cats, dogs, rabbits, monkeys and rats, for these purposes in order to develop new methods and techniques for organ and limb transplants.

These compounds (Examples 3 and 4) and their use as platelet

aggregation inhibitor drugs are believed to be unobvious because they were the most active of a series of related test peptides. To our knowledge from the initial platelet aggregation studies, these homoarginine analogs of the GRGDS cell-adhesion domain are more potent than any pentapeptide analogs known to date by us. In the known structure activity relationships of this series of peptides these homoarginine-based pentapeptides are the first discovered arginine-substitute peptides that did not produce an inactive peptide in our laboratories. For example, substitution of lysine for arginine gives an inactive peptide. No known substitutions for the middle glycine and the adjacent aspartic acid have been shown to be compatible with biological activity. Further, to our knowledge, no published structure is reported to have biological activity higher than the parent GRDGS molecule. For these reasons we believe these end product compound are unexpectedly new and useful compounds which could lead to their use as practical platelet aggregation inhibition drug compounds.

The invention is further exemplified by the following detailed examples.

Example 1 Tert-butyloxycarbonylhomoarginine.

The preparation of this protected homoarginine precursor compounds involved the use of a modification of older methods used to produce tert-butoxycarbonyl-arginine, such older methods being described for example by D. Yamashiro et al. in the J. Amer. Chem. Soc., 94, 2855-2859, (1972); by H. H. Holton et al. in the Can. J. Chem., 51, 1910-1914 (1973); by J. Ramachandran et al. in the J. Org. Chem., 27, 4006-4009 (1962); and by an Aldrich Technical Bulletin for BOC-ON, Product No. 19, 337-2.

A mixture of commercially obtained homoarginine (Har), (25 g., 0.111 mol) dissolved in 110 ml. of 50% V/V aqueous dioxane mixture and 30.14 g. (0.122 mol) of 2-(tert-butyloxycarbonylimino)-2-phenyl-acetonitrile was adjusted to pH 9.3 with 0.25M sodium hydroxide solution. Then 50 ml. (neat) of dioxane was added to balance the volume of the added sodium hydroxide solution so that the solution was about 50% V/V dioxane. The resulting reaction mixture was stirred at room temperature for 23 hours and the pH was maintained at 9.0 to 9.5. The progress of the reaction was followed by thin layer chromatography (TLC) analysis of samples of the reaction mixture on

silica gel which was eluted with a n-butanol/ethyl acetate/acetic acid/water (BuOH/EtOH/HOAc/H_2O, 1:1:1:1 V/V) followed by ninhydrin detection. When the reaction was completed, the mixture was concentrated to 300 ml. and extracted three times with diethyl ether (ether). The aqueous layer was separated and cooled to 0°C., acidified to pH 3 using 3N hydrochloric acid (HCl) and washed with 100 ml. of EtOAc. Upon standing at 10°C., the resulting aqueous layer became clear and a viscous oily material (Rf=0.7; homoarginine RF=0.4) settled to the bottom. The Nuclear Magnetic Resonance (NMR) spectrum of this oily residue was consistent with the above-titled compound. Analytical high performance liquid chromatography (HPLC) standard procedure gave a product having a retention time of 12.22 minutes. The resulting Boc-homoarginine was lyophilized and then stored as a white powder. This material was used without further purification in the following example.

Example 2 Alpha-tert-butyloxycarbonyl-homoarginine(N^G-tosyl)

This chemical intermediate was made using a modified form of the general peptides making procedures used for the preparation of Nα-tert-butyloxycarbonyl-N^G-tosyl-arginine (BOC-Arg(Tos)), described in the Yamashiro et al. J. Amer. Chem. Soc., 94, 2855-2859, the Holton et al. Can. J. Chem., 51, 1910-1913, and the Ramachandran et al. J. Org. Chem., 27, 4006-4009 references, supra.

A mixture of 17.15 g. (90 mmol.) of tosyl chloride (p-methylphenylsulfonyl chloride) in minimal acetone was added dropwise to an ice-cooled solution of tert-butyloxycarbonyl-homoarginine (BOC-Har) in 80% V/V aqueous acetone which had been pH adjusted to 12.5 with a 4N sodium hydroxide solution. The pH was maintained at 12.0 to 12.5. After three hours the resulting reaction mixture showed (by TLC procedures, using silica gel and a chloroform/methanol/acetic acid, 36:3:1 V/V mixture as eluant with ninhydrin development) a weak spot at the origin of the sample (starting material), another spot at Rf=0.22 to 0.37 and a faint spot at Rf=0.83. The reaction mixture was adjusted to pH 7 with ice-cold 3N hydrochloric acid and the acetone was removed from the mixture using a rotary evaporator. The residual aqueous solution was in turn extracted three times with ether, acidified to pH 3.5 with 3N hydrochloric acid, extracted with ethyl acetate, further acidified to pH 1.5 and extracted again with ethyl acetate. The ethyl acetate layers were combined and rotary

evaporated to remove some ethyl acetate to leave a viscous oil residue. Analytical HPLC of the oil showed a major peak at 12.9 minutes. The crude product was purified in two steps on a Waters Prep 500 HPLC machine. An initial cut was made with a prep-pak C4 (gradient 100% water to a 75/25% V/V water/acetonitrile mixture) and rechromatographed on a prep-pak C18 (gradient 75/25% to 65/35% V/V water/acetonitrile mixtures. Appropriate fractions of the eluant were pooled and concentrated to yield 4.23 g. of the above-titled product as a white glassy solid. Purity of the product was determined by using analytical HPLC standard conditions (Rt 13.32 min) and the product had an estimated purity of 92%. The major impurity by NMR spectrum analysis procedures was N^G-tosylhomoarginine [Har(Tos)], analytical HPLC Rf=9.78). The FAB/Mass Spectrum Mass Ion plus hydrogen for the titled product was 443 (mol weight 442.19).

A formal chemical name for the main product of this example is N2-[(1,1-dimethylethoxy)carbonyl]-N6-[imino[[(4-methylphenyl)sulfonyl]amino]methyl]-L-Lysine.

Example 3 Gly-Har-Gly-Asp-Ser; N-[N-[N-[N6-(Aminoiminomethyl)-N2-glycyl-L-lysyl]glycyl]-L-α-aspartyl]-L-Serine

Starting with 1.44 g. (1.04 meq./g.; 1.5 meq. of Merrifield resin, the titled peptide was elaborated using the above-outlined general peptide synthesis procedure except for the coupling of the Boc-Har-(Tos).

The coupling of the Boc-Har(Tos) from Example 1 to the glycine-cyclohexyl-protected-aspartic acid-benzyl-protected-serine-Merrifield resin substrate was done in the usual peptide synthesis manner; however, a negative ninhydrin test was not obtained after 24 hours of coupling time. A 10% V/V diisopropylethylamine in dichloromethane (DIPEA) (1.5 meq.) solution was added and the coupling was allowed to proceed for another 24 hours. A negative ninhydrin test was still not achieved. A quantitative ninhydrin test [see E. Kaiser et al., Anal. Biochem., 34, 595-598 (1970); and V. K. Sarin et al., "Quantitative Monitoring of Solid Phase Peptide Synthesis by the Ninhydrin Reaction" in Peptides: Synthesis, Structure and Function, Editors D. H. Rich and E. Gras, Pierce Chemical Co., Publisher, 221-224 (1981); and Anal. Biochem., 117, 147-157 (1981)] indicated only about 80% coupling. The remaining about 20% substrate was acetylated (capped) as described in the general peptide synthesis described hereinabove.

The remainder of the synthesis was completed without incident to give 2.62 g. of the dried intermediate peptide-resin. The titled peptide was cleaved from the resin using the general hydrofluoric acid (HF) procedure described hereinabove. Preparative HPLC purification of the resulting peptide was performed using a Michele Miller side D Vydac C-18 prep column. A three-hour gradient from 90% water/10% V/V acetonitrile to 78% water/22% acetonitrile mixture was run. The fractions were analyzed on an analytical HPLC and the appropriate fractions were pooled, rotary evaporated to dryness, re-dissolved in water and lyophilized to give 117.3 mg. of the titled pure peptide.

The purity by analytical HPLC methods was 100%. The FAB-MS mass ion plus hydrogen was 505 (molecular weight was 504.23). The amino acid analysis was: Aspartic acid (Asp (1) 1.07; Glycine (Gly) (2) 1.96; Serine (Ser)(1) 0.83; homoarginine (Har) (1) 0.98; about 79% W/W peptide.

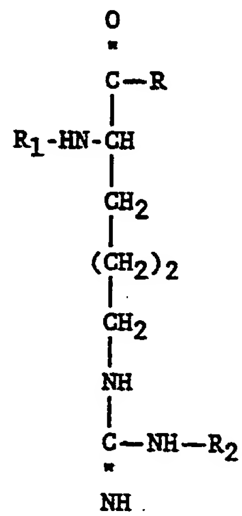
<u>Example</u> <u>4</u> N-[N-[N-[N2-(N-acetylglycyl)-N6-(aminoiminomethyl)-L-lysyl]glycyl]L-α-aspartyl]-L-serine (Ac-Gly-Har-Gly-Asp-Ser)

A 0.87 gm. portion of the Gly-Har(Tos)-Gly-Asp(Chxyl)-Ser(Bzl)-Merrifield resin, prepared as described in Example 3 hereinabove was acetylated according to the general acetylation procedure described above to give an Ac-Gly-Har(Tos)-Gly-Asp(Chxyl)-Ser(Bzl)-Merrifield resin intermediate. The titled compound was obtained from the Merrifield resin substrate by anhydrous hydrofluoric acid (HF) standard cleavage procedure. The titled compound was purified using a Michele-Miller size D Vydoc C-18 preparatory column and eluted with linear gradiant mixtures of 88/22% to 73/27% V/V water/acetonitrile over three hours. The fractions containing the purified titled compound were pooled, rotary evaporated to dryness, re-dissolved in water and lyophilized to give 89.5 mg. of the titled acetylated peptide which gave only one peak on analytical HPLC using our above standard gradient (Rt-6.26).

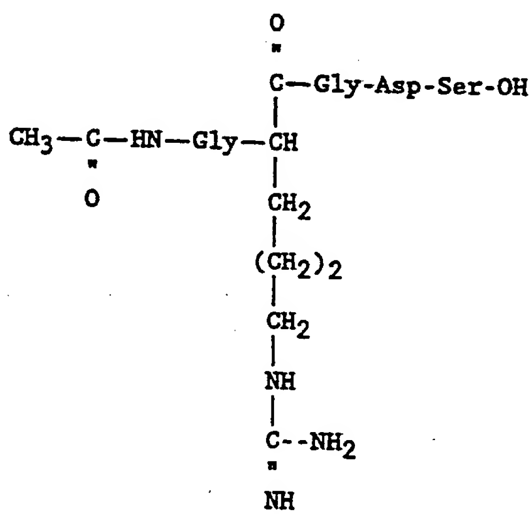
The purity of the title acetylated peptide by analytical HPLC was 100%. The Mass Spectrum (MS) analysis-FAB Mass ion plus hydrogen was 547 (molecular weight 546.24).

The amino acid analysis was: Asp (1) 106; Gly (2) 2.01; Ser (1) 0.78; Har (1) 0.93; about 66% peptide.

STRUCTURES

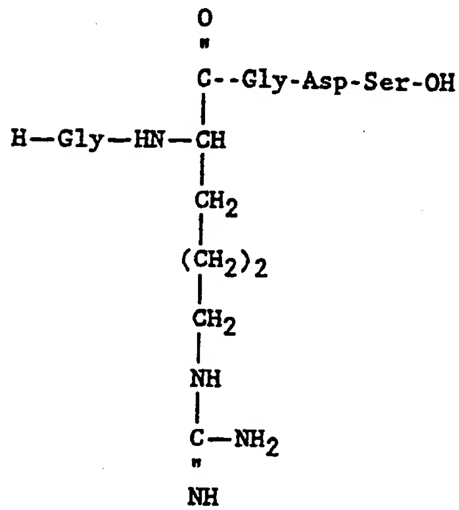


(I)

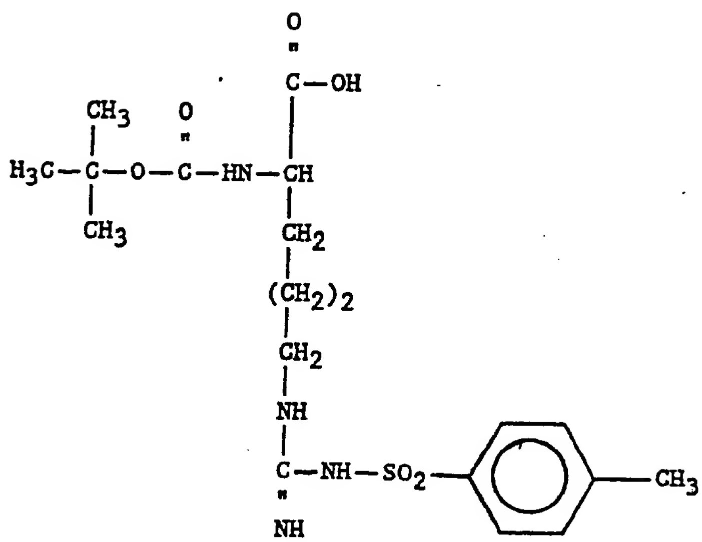


(II)

STRUCTURES (CONTINUED)

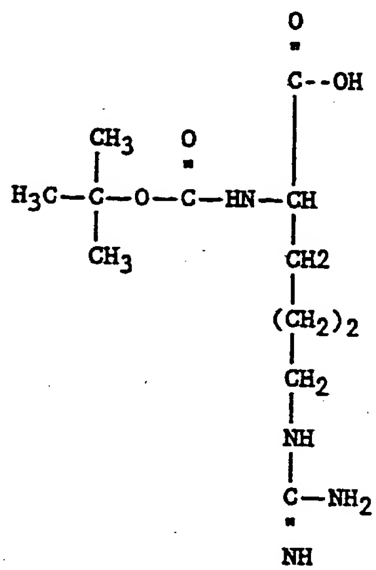


(III)



(IV)

STRUCTURES (CONTINUED)



(V)

PROCESS

OUTLINE

HOMOARGININE

↓

(1) T-Boc-HOMOARGININE

↓

(2) T-Boc-HOMOARGININE-(N^G-TOSYL)

↓

(TO MERRIFIELD RESIN)

↓

(3) Gly-Har-Gly-Asp-Ser-Resin

↓

(optional)

[Ac-Gly-Har-Gly-Asp-Ser-Resin]

↓

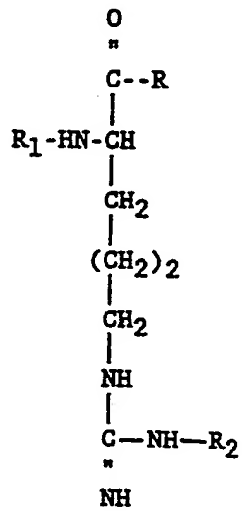
(5) (HF Treatment to remove polypeptide from resin)

↓

(6) Ac-Gly-Har-Gly-Asp-Ser-OH

CLAIMS

1. A compound of the formula



(I)

where R is selected from the group consisting of

-Gly-Asp-Ser-OH, or

-OH,

R_1 is H-Gly or C_1 to C_3-alkenyl-C(O)-Gly; and

R_2 is hydrogen, or an acid addition salt thereof.

2. A compound according to Claim 1 wherein

R is Gly-Asp-Ser-OH;

R_1 is H-Gly; and

R_2 is hydrogen, so that the compound is N-[N-[N-[N6-(aminoiminomethyl)-N2-glycyl-L-lysyl]glycyl]-L-α-aspartyl-L-Serine, or a pharmaceutically acceptable salt thereof.

3. A compound according to Claim 1 wherein

R is Gly-Asp-Ser-OH,

R_1 is CH_3-C(O)-Gly, and

R_2 is hydrogen,

so that the compound is N-[N-[N-[N2-(N-acetylglycyl)-N6-(aminoiminomethyl)-L-lysyl]glycyl]-L-α-aspartyl]-L-Serine, or a pharmaceutically acceptable salt thereof.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/00163

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 07 K 5/00; C 07 K 7/06; A 61 K 37/64

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C 07 K; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Chemical Abstracts, vol. 93, 1980 (Columbus, Ohio, US) Yokosawa, Hideoshi et al.: "The isolation of biologically-active peptides by means of immobilized anhydrotrypsin", see page 3090, abstract no. 234173f & Pept. Chem. 1976, (Pub. 1977) 14th, 147-50	1
	--	
Y	Chemical Abstracts, vol. 89, 1978 (Columbus, Ohio, US) B. Moore et al.: "Effect of the basic amino acid side chain lenght and the penultimate residue on the hydrolysis of benzoyldipeptides by carboxypeptidase B", see page 329, abstract no. 102654w & Can. J. Biochem. 1978, 56(5), 315-18	1
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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd April 1989	24. 04. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN